

Comparison of Phenanthrene and Pyrene Degradation by Different Wood-Decaying Fungi

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The degradation of phenanthrene and pyrene was investigated by using five different wood-decaying fungi. After 63 days of incubation in liquid culture, 13.8 and 4.3% of the [ring U-¹⁴C]phenanthrene and 2.4 and 1.4% of the [4,5,9,10-¹⁴C]pyrene were mineralized by *Trametes versicolor* and *Kuehneromyces mutabilis*, respectively. No ¹⁴CO₂ evolution was detected in either [¹⁴C]phenanthrene or [¹⁴C]pyrene liquid cultures of *Flammulina velutipes*, *Laetiporus sulphureus*, and *Agrocybe aegerita*. Cultivation in straw cultures demonstrated that, in addition to *T. versicolor* (15.5%) and *K. mutabilis* (5.0%), *L. sulphureus* (10.7%) and *A. aegerita* (3.7%) were also capable of mineralizing phenanthrene in a period of 63 days. Additionally, *K. mutabilis* (6.7%), *L. sulphureus* (4.3%), and *A. aegerita* (3.3%) mineralized [¹⁴C]pyrene in straw cultures. The highest mineralization of [¹⁴C]pyrene was detected in straw cultures of *T. versicolor* (34.1%), which suggested that mineralization of both compounds by fungi may be independent of the number of aromatic rings. Phenanthrene and pyrene metabolites were purified by high-performance liquid chromatography and identified by UV absorption, mass, and ¹H nuclear magnetic resonance spectrometry. Fungi capable of mineralizing phenanthrene and pyrene in liquid culture produced enriched metabolites substituted in the K region (C-9,10 position of phenanthrene and C-4,5 position of pyrene), whereas all other fungi investigated produced metabolites substituted in the C-1,2, C-3,4, and C-9,10 positions of phenanthrene and the C-1 position of pyrene.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants found in air, soil, freshwater, and marine environments (10). Their distribution and fate in the environment are of increasing interest because compounds with four or more aromatic rings are recalcitrant, often carcinogenic, mutagenic, or toxic, and pose a serious risk to human health (3). Due to the high cost of trapping or removing PAHs from the environment, the potential of microorganisms for decontamination and detoxification of PAH-polluted sites is under investigation (43). Whereas the PAH mineralization and degradation pathways of bacteria have been well studied, knowledge of similar activities in fungi is limited to various white rot fungi and a few species of soil fungi (8, 12, 25). The white rot fungus *Phanerochaete chrysosporium* has been used for the investigation of PAH transformation by fungi due to extracellular enzyme activities like those of ligninase (also called lignin peroxidase [LiP]) or manganese peroxidase (MnP) (29), whereas the nonligninolytic fungus *Cunninghamella elegans* has been used as a model organism, since it can metabolize a wide variety of PAHs, like mammals (8, 11, 12). Only limited data are available about the role of brown rot and litter-decaying basidiomycetes in the metabolism of PAHs (24, 32, 38).

Phenanthrene and pyrene are two of the most abundant PAHs in the environment (8). Although they are not genotoxic, both substances have been used as indicators for monitoring PAH-contaminated wastes, since the molecular structures of phenanthrene and pyrene are found in potent carcinogenic PAHs (30). Both substances are distinguished by

their water solubility (phenanthrene, 1.3 mg/liter; pyrene, 0.14 mg/liter [9]) and their ionization potential (IP) (i.e., the energy required to remove an electron and form PAH cation radicals). Phenanthrene has an IP of 8.19 eV and is not a substrate of LiP, whereas pyrene, with an IP of 7.50 eV, is a known substrate of LiP (13).

In this study we used phenanthrene and pyrene as model PAHs for the investigation of degradation and identification of metabolites by different wood-decaying fungi. We investigated the mineralization of these substances, and we report the isolation of different chemical intermediates of their degradation by five different fungi. The aim of this study was to broaden the knowledge of PAH mineralization by wood-decaying fungi.

MATERIALS AND METHODS

Microorganisms. *Trametes versicolor* (L.:Fr.) Pilat, PKS T 08-1, and *Laetiporus sulphureus* (Bull.:Fr.) Murrill, DSM 11211, were obtained from German type collections in Weimar (Pilzkultursammlung [PKS]) and Braunschweig (Deutsche Sammlung von Mikroorganismen [DSM]), respectively. *Kuehneromyces mutabilis* (Schff.:Fr.) Sing. & A. H. Smith, DSM 11212, *Flammulina velutipes* (Curt.:Fr.) Karst., DSM 9614, and *Agrocybe aegerita* (Brig.) Sing., DSM 9613, were isolated from plectenychma of fruiting bodies. Stock cultures were maintained on malt agar slants at 4°C. Subcultures were made every 3 months.

Chemicals. [ring U-¹⁴C]Phenanthrene (117 mCi/mmol; radiochemical purity after purification by thin layer chromatography in benzene, 96.5%, determined by high-performance liquid chromatography [HPLC] analysis) and [4,5,9,10-¹⁴C]pyrene (56 mCi/mmol; radiochemical purity, 98%) were purchased from Amersham Buchler (Braunschweig, Germany). Phenanthrene, pyrene, 9-phenanthrol, and 1-pyrenol were obtained from Aldrich (Steinheim, Germany). All solvents were of HPLC grade, and all other chemicals were of the highest purity available from Merck (Darmstadt, Germany), Fluka Chemie AG (Neu Ulm, Germany), and Fisher Chemical Co. (Pittsburg, Pa.).

Mineralization of PAH in liquid culture and detection of metabolites. Kinetic studies of [¹⁴C]phenanthrene and [¹⁴C]pyrene degradation were conducted in triplicate in 300-ml Erlenmeyer flasks containing 100 ml of nutrient-nitrogen-limited medium (pH 4.5) prepared according to the method of Kirk et al. (19) with 10 g of glucose/liter and 1.2 mM ammonium tartrate and inoculated with

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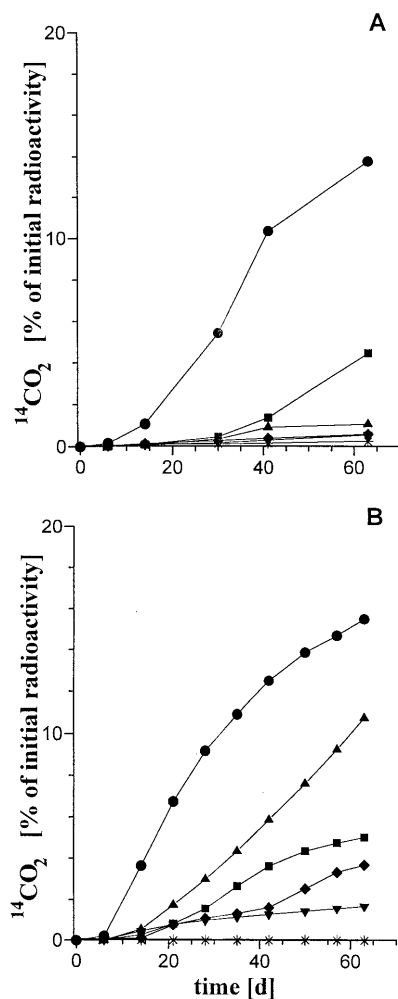


FIG. 1. Mineralization of [^{14}C]phenanthrene in liquid (A) and straw (B) cultures by *T. versicolor* (●), *K. mutabilis* (■), *L. sulphureus* (▲), *F. velutipes* (▼), and *A. aegerita* (◆); (*) represents abiotic controls. The means of triplicate determinations are given (standard deviation <1.5%).

15-mycelium 1-cm² plugs of malt agar cultures. After 48 h, 100 μl of phenanthrene or pyrene solution (1 μCi of radiolabelled and unlabelled PAH with a final concentration of 5 mg/100 ml, dissolved in dimethylformamide) was added to each sample. Fungi were incubated in the dark at 24°C. Cultures were continuously flushed with sterile, CO₂-free, humidified air. The air was passed through dimethylformamide to trap volatile organic substances. The liberated ¹⁴CO₂ was trapped in 2 N NaOH and quantified as described previously (35). Sterile controls were prepared to detect abiotic PAH degradation. For mass balance analysis, culture fluid and mycelia were extracted as described below after 63 days of incubation. After extraction, mycelia were air dried, homogenized, and combusted as described previously (35).

To identify phenanthrene and pyrene metabolites, fungi were cultivated in 1,000-ml Erlenmeyer flasks containing 400 ml of nutrient-nitrogen-limited medium prepared according to the method of Kirk et al. (19) and inoculated with 50-mycelium plugs. Phenanthrene or pyrene (20 mg) was dissolved in 500 μl of dimethylformamide and added to one flask after 48 h of incubation (final concentration, 50 mg/liter). After 63 days of incubation the culture fluid and mycelia were extracted with ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure at 40°C. The residue was dissolved in methanol and analyzed by HPLC with a model 1040A diode array detector (Hewlett-Packard, Palo Alto, Calif.) or a model L 4500 diode array detector (Merck Hitachi) operating at 254 nm. A 5- μm RP 18 column (250 by 4.6 mm inner diameter) (Spherisorb ODS-2 [Metachem Technologies, Torrance, Calif.] or LiChrospher 100 [Merck]) was used to separate phenanthrene and pyrene metabolites by using a 40-min programmed methanol-water linear gradient (50 to 95%, vol/vol) with a flow rate of 1 ml/min. Metabolites were collected after repeated injections of the culture extracts, and fractions with similar compositions and HPLC retention times were pooled and

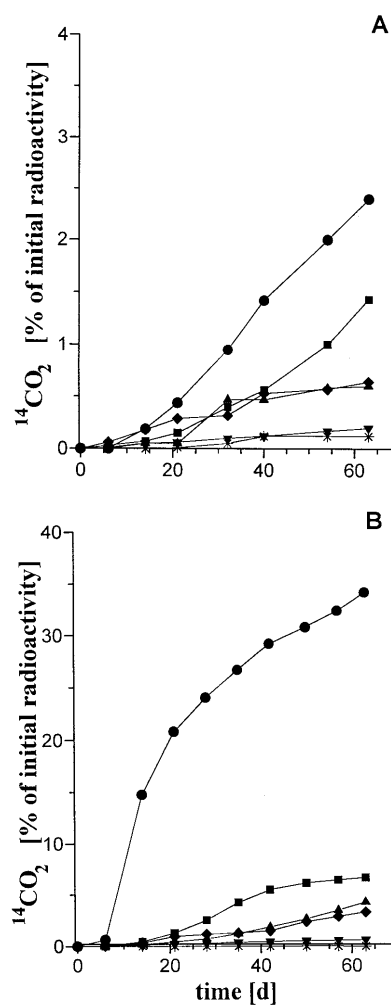


FIG. 2. Mineralization of [^{14}C]pyrene in liquid (A) and straw (B) cultures by *T. versicolor* (●), *K. mutabilis* (■), *L. sulphureus* (▲), *F. velutipes* (▼), and *A. aegerita* (◆); (*) represents abiotic controls. The means of triplicate determinations are given (standard deviation <1.5%).

concentrated in a Speed Vac concentrator (Savant Instruments, Hicksville, N.Y.). To analyze ¹⁴C-labelled metabolites, 1-ml fractions were collected every minute in scintillation vials and mixed with 13 ml of scintillation cocktail (35) and the radioactivity of each fraction was determined by liquid scintillation counting. Gas chromatography-mass spectrometry and ¹H nuclear magnetic resonance (¹H-NMR) analyses were performed as described previously (34).

Mineralization of PAH in straw culture. Kinetic studies of [^{14}C]phenanthrene and [^{14}C]pyrene mineralization in straw cultures were conducted in triplicate over a period of 63 days as described previously (21, 26) in 300-ml Erlenmeyer flasks containing 5 g (dry weight) of milled wheat straw (particle size, <1 mm; sterilized at 121°C for 40 min). Ethanolic phenanthrene or pyrene solution (250 μl ; containing 0.5 μCi of radiolabelled and unlabelled PAH with a final concentration of 5 mg/5 g of straw) was suspended in 10 ml of sterilized water and added to each sample under sterile conditions. Since the straw was supplied with 5 ml of water before autoclaving, the final water content of the substrate was approximately 75%. After inoculation with two-mycelium 1-cm² plugs from malt agar cultures, the flasks were aerated continuously with CO₂-free air, and the CO₂ produced was trapped in 25 ml of 2 N NaOH and analyzed as described previously (26). Sterilized straw samples without fungi served as controls.

Enzyme assays. To determine the production of the extracellular ligninolytic enzymes MnP, LiP, and laccase, fungi were cultivated in 300-ml Erlenmeyer flasks as described above for mineralization experiments. One-milliliter samples of culture broth were taken under sterile conditions, centrifuged, and analyzed. MnP was assayed directly by the formation of Mn³⁺-malonate complexes as described by Wariishi et al. (42). Laccase activity was measured with ABTS [2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid)] as a substrate according to the method of Wolfenden and Willson (44), and LiP activity was determined by the veratryl alcohol oxidation method at pH 3.0 according to the method of Tien and

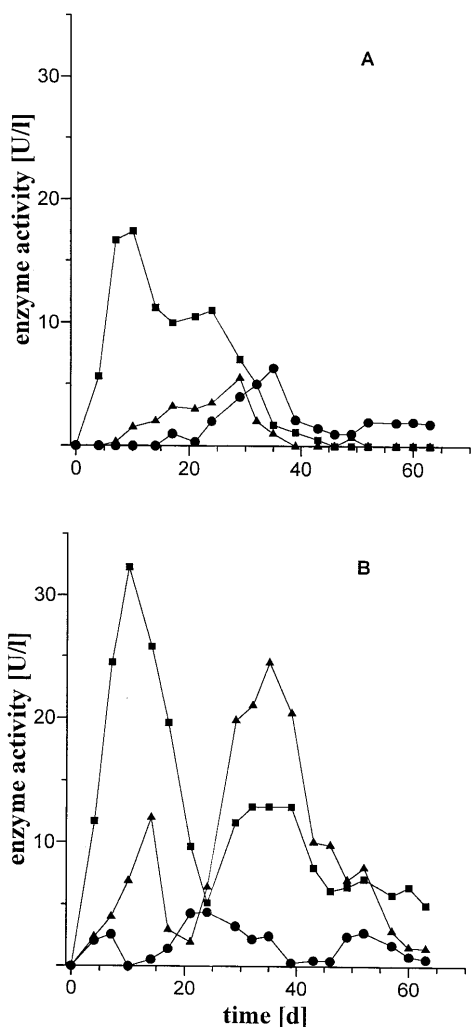


FIG. 3. Production of the extracellular ligninolytic enzymes MnP (▲), LiP (●), and laccase (■) by *T. versicolor* (A) and *K. mutabilis* (B) during cultivation in liquid cultures.

Kirk (39). All activities were expressed in units defined as follows: 1 U = 1 μmol of substrate oxidized per minute at 25°C.

RESULTS

Mineralization of phenanthrene and pyrene. Fungi were incubated with 50 mg of phenanthrene or pyrene/liter under

low-nitrogen conditions according to the method of Kirk et al. (19) with 10 g of glucose/liter for 63 days. Low-nitrogen cultures were chosen, since Hammel et al. (14) demonstrated an enhancement in the mineralization of phenanthrene by *P. chrysosporium* in low-nitrogen cultures in comparison to that in high-nitrogen cultures. The experiments were conducted in triplicate, and the standard deviation of all values was less than 1.5%. Figure 1A shows that the white rot fungi *T. versicolor* and *K. mutabilis* mineralized 13.8 and 4.3% of the [^{14}C]phenanthrene, respectively. A conspicuously lower mineralization of 2.4 and 1.4% by *T. versicolor* and *K. mutabilis*, respectively, was detected in liquid cultures incubated with [^{14}C]pyrene (Fig. 2A). No (<1%) $^{14}\text{CO}_2$ was released in cultures of the wood-decaying fungi *F. velutipes*, *L. sulphureus*, and *A. aegerita* or in sterile controls during phenanthrene and pyrene degradation (Fig. 1A and 2A).

The production of the extracellular ligninolytic enzymes MnP, LiP, and laccase is shown in Fig. 3. While the white rot fungi *T. versicolor* (Fig. 3A) and *K. mutabilis* (Fig. 3B) were able to excrete those enzymes into the culture broth, no enzyme activities were detected for the other wood-decaying fungi investigated.

To investigate the mineralization of phenanthrene and pyrene by wood-decaying fungi grown on their natural substrate, PAHs were adsorbed on wheat straw. The white rot fungi *T. versicolor* and *K. mutabilis* mineralized 15.5 and 5.0% of phenanthrene, respectively, in a period of 63 days (Fig. 1B). In contrast to their lack of activity in liquid media, *L. sulphureus* and *A. aegerita* were able to mineralize 10.7 and 3.7% of phenanthrene, respectively (Fig. 1B). The highest mineralization of [^{14}C]pyrene (Fig. 2B) was detected in straw cultures of *T. versicolor* (34.1%). Lower values were detected for *K. mutabilis* (6.7%), *L. sulphureus* (4.3%), and *A. aegerita* (3.3%). No mineralization (1.7 and 0.6%) was detectable in cultures of *F. velutipes* (Fig. 1B and 2B). In sterile controls 0.2% of $^{14}\text{CO}_2$ was liberated from phenanthrene and pyrene. The standard deviation of all values was less than 1.5%.

Mass balance of radioactive carbon in liquid cultures. The mass balance analyses of [^{14}C]phenanthrene and [^{14}C]pyrene are summarized in Tables 1 and 2, respectively. The total radioactivity recovered was determined by summing all the individual values of (i) liberated $^{14}\text{CO}_2$, (ii) radioactivity in ethyl acetate extracts of culture fluid and mycelia, (iii) non-ethyl acetate-extractable radioactivity, (iv) radioactivity adsorbed by mycelia, and (v) volatile organic substances and was expressed as the percentage of the radioactivity initially added. Total recovery ranged from 91.7 to 98.9% for phenanthrene and from 90.0 to 98.6% for pyrene. The standard deviation of all individual values was less than 3%.

In phenanthrene degradation, more than 19.0% of the con-

TABLE 1. Mass balance analysis, after 63 days, of radioactivity (1 μCi) initially added as [^{14}C]phenanthrene to liquid cultures of wood-decaying fungi

Source of radioactivity	Radioactivity (%) ^a					
	Control	<i>T. versicolor</i>	<i>K. mutabilis</i>	<i>L. sulphureus</i>	<i>F. velutipes</i>	<i>A. aegerita</i>
$^{14}\text{CO}_2$	0.3	13.8	4.3	1.1	0.6	0.7
Volatile organic substances	19.4	5.0	10.8	8.5	10.7	4.1
Ethyl acetate extract	70.1	37.3	50.6	60.6	46.5	59.8
Aqueous phase	2.4	19.9	9.9	7.5	5.0	2.4
Mycelia	0	22.9	18.9	14.9	28.9	25.9
Balance	92.2	98.9	94.5	92.6	91.7	92.9

^a All values are means ($n = 3$) with a standard deviation of <3%.

TABLE 2. Mass balance analysis, after 63 days, of radioactivity (1 μ Ci) initially added as [14 C]pyrene to liquid cultures of wood-decaying fungi

Source of radioactivity	Radioactivity (%) ^a					
	Control	<i>T. versicolor</i>	<i>K. mutabilis</i>	<i>L. sulphureus</i>	<i>F. velutipes</i>	<i>A. aegerita</i>
¹⁴ CO ₂	0.1	2.4	1.4	0.6	0.2	0.8
Volatile organic substances	1.1	0.5	0.2	0.5	0.3	0.7
Ethyl acetate extract	97.3	39.9	60.8	81.0	61.7	60.0
Aqueous phase	0.1	3.3	2.6	1.6	0.6	2.9
Mycelia	0	43.9	29.2	9.0	28.8	27.9
Balance	98.6	90.0	94.2	92.7	91.6	92.3

^a All values are means ($n = 3$) with a standard deviation of <3%.

control radioactivity and between 4.1 and 10.8% of the fungal culture radioactivity was volatile and was identified as phenanthrene by HPLC.

After extracting the culture fluid and mycelia with ethyl acetate, the extracted mycelia were combusted. Independently of biomass (final dry weights [g/liter]: *T. versicolor*, 3.7; *K. mutabilis*, 5.5; *F. velutipes*, 3.5; *L. sulphureus*, 2.2; *A. aegerita*, 3.8), high proportions (9.0 to 43.9%) of the radioactivity initially added were associated with mycelia. Most of this was probably due to unreacted phenanthrene and pyrene. The hydrophobic nature of phenanthrene and pyrene indicates their affinity for accumulation in biological materials. The values are comparable to those previously described for other fungi cultivated with phenanthrene or pyrene (20, 34, 46). In cultures with fungi, 37.3 to 60.6% (phenanthrene) and 46.9 to 81.0% (pyrene) of the total radioactivity was extractable. The distribution of unmetabolized parent compounds, polar metabolites, and nonpolar metabolites (probably products of PAH polymerization) of phenanthrene and pyrene, expressed as percentages of the ethyl acetate-extractable radioactivity, are shown in Tables 3 and 4. The polar metabolites that have been identified are described below, and identification of metabolites with retention times higher than those of phenanthrene and pyrene is under way. A total of 2.4 to 19.9% (phenanthrene) and 0.2 to 3.1% (pyrene) of radioactivity was nonextractable in cultures with fungi and in controls. In comparison with controls, *T. versicolor* decreased the extractable fraction in phenanthrene and pyrene samples most of all. The nonextractable fraction of phenanthrene samples was increased ~15% (Table 3) by this fungus, indicating that polar, water-soluble metabolites were formed. Lange et al. (23, 24) demonstrated the formation of acetate as an intermediate in pyrene degradation by the litter-decaying fungus *Crinipellis stipitaria*. It is conceivable that such low-molecular-weight metabolites are responsible for the increased radioactivity in the nonextractable fraction. Further research is in progress to identify these intermediates.

Identification of ethyl acetate-extractable metabolites of phenanthrene and pyrene metabolism. After 63 days of incubation, culture fluid and mycelia were separated and extracted with ethyl acetate. Both extracts were combined, and the major metabolites were characterized by a comparison of their relative HPLC retention times and UV-visible absorption and mass spectra to previously published spectra and synthetic standards.

A major metabolite detected in phenanthrene degradation by *T. versicolor*, *K. mutabilis*, *F. velutipes*, and *A. aegerita* had a HPLC retention time of 10.9 min. The mass spectrum (Table 5) was comparable to that published for phenanthrene 9,10-dihydrodiol (1, 37). ¹H-NMR spectroscopy analysis was con-

ducted to determine the configuration of this metabolite (Table 6). The chemical shifts and coupling constants of the metabolite were similar to those previously reported for the *trans* isomer, the major metabolite of the white rot fungus *P. chrysosporium* (37). Furthermore, in phenanthrene cultures, phenanthrene *trans*-1,2-dihydrodiol and phenanthrene *trans*-3,4-dihydrodiol were produced by *L. sulphureus* and *A. aegerita*, respectively. The identities of both dihydrodiols were confirmed by HPLC retention time, UV absorption, mass spectrometry (Table 5), and ¹H-NMR spectrometry (Table 6). In addition to dihydrodiols, phenanthrene degradation produced monohydroxyl metabolites. The mass spectra of the metabolites produced by *F. velutipes*, *L. sulphureus*, and *A. aegerita* showed a molecular ion (M^{+}) at m/z 194 and fragment ions at m/z 165, typical of a phenanthrol (7). The ¹H-NMR spectral analysis of the phenanthrol formed by *F. velutipes* and *L. sulphureus* confirmed the site of hydroxyl substitution as the C-2 position on phenanthrene (Table 6). Phenanthrene monohydroxylated in the C-1 position (*F. velutipes* and *L. sulphureus*) and the C-3 and C-4 positions (*A. aegerita*) was detected in ethyl acetate extracts, in comparison with metabolites identified for *Aspergillus niger* and *Syncephalastrum racemosum*, as described recently (7, 34), and by ¹H-NMR analyses (Table 6).

Pyrene degradation by *T. versicolor*, *K. mutabilis*, and *A. aegerita*, in contrast to that of soil fungi investigated (34), produced a major HPLC peak at 12.5 min and had a UV absorption and mass spectrum similar to those shown for pyrene *trans*-4,5-dihydrodiol in previous studies (2, 15, 22). While 1-pyrenol was produced by *A. aegerita* in addition to pyrene-4,5-dihydrodiol as a second major metabolite, it occurred as minor peaks in cultures of *K. mutabilis* and *L. sulphureus*. 1-Pyrenol had a HPLC retention time, UV absorption, and

TABLE 3. Percentage of ethyl acetate-extractable metabolites and unmetabolized parent compound after 63-day incubation of fungi with [14 C]phenanthrene

Organism	Fraction (%)		
	Polar metabolites (0–33 min) ^a	Phenanthrene (33 min)	Less polar metabolites (34–55 min)
<i>T. versicolor</i>	34.7	62.2	3.1
<i>K. mutabilis</i>	21.1	76.0	2.9
<i>F. velutipes</i>	27.1	68.5	4.4
<i>L. sulphureus</i>	57.3	38.7	4.0
<i>A. aegerita</i>	22.3	59.2	18.5
Control	0.3	96.5	3.2

^a HPLC retention time.

TABLE 4. Percentage of ethyl acetate-extractable metabolites and unmetabolized parent compound after 63-day incubation of fungi with [¹⁴C]pyrene

Organism	Fraction (%)		
	Polar metabolites (0–39 min) ^a	Pyrene (39 min)	Less polar metabolites (40–55 min)
<i>T. versicolor</i>	22.3	58.7	19.0
<i>K. mutabilis</i>	62.9	21.6	15.5
<i>F. velutipes</i>	29.3	56.6	14.1
<i>L. sulphureus</i>	76.6	14.9	8.5
<i>A. aegerita</i>	48.0	35.1	16.9
Control	0.0	98.0	2.0

^a HPLC retention time.

mass spectrum similar to those published previously (34) and was consistent with synthetic 1-pyrenol.

Additionally, pyrene degradation by *L. sulphureus* produced a metabolite with a retention time of 13.7 min. It had a mass spectrum with a molecular ion (M⁺) at *m/z* 236 and fragment ions at *m/z* 218 (M⁺—H₂O) and *m/z* 189 (M⁺—H₂O—·HCO), characteristic of a dihydrodiol (22). The UV spectrum, with λ_{max} at 218, 232, 266, 281, 297, 309, 352, and 369 nm, is different from that of pyrene-4,5-dihydrodiol identified in pyrene degradation by *T. versicolor*, *K. mutabilis* (as described above), *Pleurotus ostreatus* (2), and *C. stipitaria* (22). ¹H-NMR analysis was not successful, and the identity of this metabolite is still unknown.

DISCUSSION

Our studies have confirmed that wood-decaying fungi are able to mineralize [¹⁴C]phenanthrene and [¹⁴C]pyrene in liquid and solid (straw) cultures. The values of phenanthrene mineralization in liquid cultures by the white rot fungi *T. versicolor* and *K. mutabilis* are comparable to those published for other white rot fungi. According to Bumpus (6), *P. chrysosporium* was able to convert 7.7% of the radioactivity in [9-¹⁴C]phenanthrene to ¹⁴CO₂ in 27 days, and Morgan et al. (28) reported that *P. chrysosporium* mineralized 1.3% of the phenanthrene to ¹⁴CO₂ in 70 days. The comparison of ligninolytic enzyme activities in noninduced cultures of *T. versicolor* and *K. mutabilis* demonstrates similar activities, with those of the white rot fungus *Nematoloma frowardii* (17) capable of mineralizing PAHs (35). As recently described, MnP was able to

metabolize (4, 27, 36) and was capable of mineralizing phenanthrene and pyrene in the presence of appropriate mediator substances (36). Hammel et al. (13) and Vazquez-Duhalt et al. (41) demonstrated the role of LiP in initial pyrene oxidation by *P. chrysosporium*.

A comparison of phenanthrene and pyrene mineralization in straw cultures of *T. versicolor* indicated a higher mineralization of pyrene than of phenanthrene. Adsorption of PAHs on straw makes PAH more convenient and obtainable for mineralization. It could be that growth on straw more effectively stimulated the production of appropriate enzymes. The simultaneous degradation of straw and PAH by white rot fungi was demonstrated by In der Wiesche et al. (18) and Lang et al. (21) with [¹⁴C]pyrene as the model substance. Lang et al. (21) cultivated *P. ostreatus* in the same system that was used for our degradation studies. They followed the straw degradation as loss of lignin and as loss of organic matter and found a correlation between the time courses of straw degradation and pyrene mineralization. Furthermore, they compared extractable MnP and laccase activities in liquid and straw cultures and detected higher enzyme activities in straw cultures. The highest rate of pyrene mineralization occurred during days 20 to 45 (a period of high MnP activity), suggesting a role for MnP in PAH degradation. The additional activity of fungal LiP may be responsible for the increased mineralization of pyrene by *T. versicolor*. To our knowledge, the results of phenanthrene and pyrene mineralization provide the first evidence that the extent of PAH mineralization by fungi may be independent of the number of aromatic rings, if bioavailability is comparable.

In contrast to liquid cultures, straw stimulated phenanthrene and pyrene mineralization in *L. sulphureus* and *A. aegerita* incubations. Mineralization experiments were conducted in liquid cultures at pH 4.5. In this case, no extracellular oxidase or peroxidase activity was detectable for either fungus (results not shown). Ritschkoff and Viikari (31) suggested that the structure and chemical conformation of cellulose act as inducers for hydrogen peroxide production by brown rot fungi. Furthermore, Hirano et al. (16) described the capability of brown rot fungi to produce H₂O₂ and then to reduce the H₂O₂ to highly reactive hydroxyl radicals. Besides radicals and intracellular enzymes, it is conceivable that enzymes with pH optima other than pH 4.5 were active in PAH degradation in straw cultures. This hypothesis is supported by the investigations of Upadhyay (40), who detected activity of a peroxidase at pH 7.0 for *A. aegerita* and the ability of that peroxidase to oxidize veratryl alcohol (the substrate of LiP). PAHs were also oxidized at pH

TABLE 5. Mass spectral data for metabolites obtained from phenanthrene and pyrene metabolism by wood-decaying fungi

Compound	HPLC retention time (min)	Mass spectrum (electron ionization) ^a
Phenanthrene <i>trans</i> -1,2-dihydrodiol	10.8	212 (51), 194 (56), 181 (12), 168 (24), 166 (100), 165 (85), 152 (22), 140 (24), 128 (7), 127 (8), 115 (8), 97 (8), 83 (16), 82 (30), 76 (8), 69 (11)
Phenanthrene <i>trans</i> -3,4-dihydrodiol	4.7	212 (73), 194 (95), 181 (12), 168 (100), 166 (94), 165 (99), 152 (26), 140 (50), 128 (10), 127 (11), 115 (13), 97 (10), 83 (41), 82 (24), 70 (19)
Phenanthrene <i>trans</i> -9,10-dihydrodiol	10.9	212 (100), 194 (46), 181 (92), 166 (51), 165 (95), 152 (23), 139 (5), 115 (8), 83 (21), 82 (28), 77 (10)
1-Phenanthrol	23.1	194 (100), 166 (21), 165 (77), 139 (5), 115 (3), 97 (14), 83 (13), 82 (39), 69 (10)
2-Phenanthrol	21.5	194 (100), 166 (8), 165 (36), 139 (5), 115 (2), 97 (20), 83 (7), 82 (26), 69 (7)
3-Phenanthrol	22.0	194 (100), 166 (7), 165 (35), 139 (4), 115 (2), 97 (17), 82 (27), 70 (7)
4-Phenanthrol	23.5	194 (100), 166 (27), 165 (59), 139 (5), 115 (3), 97 (10), 83 (16), 82 (29), 69 (10)
Pyrene-4,5-dihydrodiol	12.5	236 (76), 219 (43), 218 (100), 205 (17), 201 (13), 191 (18), 190 (18), 189 (64), 178 (11), 176 (13), 118 (5), 109 (6), 100 (8), 95 (83), 94 (26), 88 (13)
1-Pyrenol	27.8	218 (100), 190 (15), 189 (60), 187 (10), 163 (3), 109 (14), 100 (2), 95 (71), 94 (21), 81 (9)

^a Characteristic ions observed with relative abundance, *m/z* (percent).

TABLE 6. 500-MHz ¹H-NMR spectral parameters of metabolites formed from phenanthrene and pyrene by wood-decaying fungi^a

Compound	Proton assignment (ppm)										Coupling constant (Hz)											
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	J _{1,2}	J _{1,3}	J _{2,3}	J _{2,4}	J _{3,4}	J _{4,5}	J _{5,6}	J _{5,7}	J _{6,7}	J _{6,8}	J _{7,8}	J _{9,10}
Phenanthrene <i>trans</i> -1,2-dihydrodiol	4.87	4.47	6.20	7.25	8.20	7.52	7.47	7.87	7.82 ^b	7.85 ^b	11.6	2.4	11.6	2.6	10.3	8.8	1.3	6.9	1.5	8.2	8.6	
Phenanthrene <i>trans</i> -3,4-dihydrodiol	6.70	6.23	4.37	5.37	8.31	7.55	7.45	7.85	7.82	7.35	9.5	9.5	5.4	1.3	1.8	8.8	1.3	6.9	1.5	8.2	8.6	
Phenanthrene <i>trans</i> -9,10-dihydrodiol	7.70	7.34	7.36	7.79	7.79	7.36	7.34	7.70	4.61	4.61	7.3	1.9	7.5	1.7	7.5	7.1	1.7	7.5	1.9	7.3		
1-Phenanthrol		7.10	7.48	8.28	8.74	7.65	7.61	7.94	7.76	8.22			7.7	0.9	8.2	8.2	1.3	7.7	1.5	8.0	9.0	
2-Phenanthrol	7.31		7.26	8.65	8.66	7.61	7.51	7.88	7.73	7.65		2.6			8.8	8.4	1.1	7.1	1.5	8.0	8.8	
3-Phenanthrol	7.82	7.22		8.12	8.61	7.61	7.58	7.90	7.60	7.71	8.6		7.7	2.4		8.0	1.3	7.1	1.5	7.7	8.8	
4-Phenanthrol	7.48	7.43	7.21		9.82	7.61	7.57	7.91	7.76	7.73	7.5	1.5				8.4	1.5	7.1	1.7	7.7	9.0	
1-Pyrenol		7.62	8.10	8.00	7.91	8.12	7.96	8.14	8.05	8.42			8.2			9.0					7.7	9.0

^a All samples were dissolved in acetone-d₆.^b Assignments may be reversed.

7.0 (36a) by that peroxidase. These results were unexpected. Further investigations will clarify the effect.

trans-Dihydrodiols and monohydroxylated phenanthrene and pyrene were the major metabolites in liquid cultures. Bezalel et al. (1) identified phenanthrene *trans*-9,10-dihydrodiol in neutral and 2,2-diphenic acid in acidified ethyl acetate extracts as metabolites of the white rot fungus *P. ostreatus*. Hammel et al. (14) reported that phenanthrene *trans*-9,10-dihydrodiol was persistent in N-limited fungal cultures and that only under nonligninolytic conditions were ring fission products like 2,2-diphenic acid detected. In all ethyl acetate extracts investigated, no significant amounts of phenanthrene quinones or pyrene quinones were present. Similarly, Bogan and Lamar (5) reported a degradation of phenanthrene by *Phanerochaete laevis*, with no significant accumulation of quinone intermediates, in a period of four weeks.

Phenanthrene and pyrene *trans*-dihydrodiols are suspected to originate from the activity of cytochrome P-450 monooxygenases in conjunction with epoxide hydrolase (1, 2), an enzymatic system that oxidizes PAHs at different sites. It is obvious that the white rot fungi enriched dead-end products of phenanthrene degradation transformed in the C-9,10 position (K region), whereas all other fungi investigated reacted like soil fungi and produced metabolites substituted in the C-1,2, C-3,4, and C-9,10 positions. Pyrene degradation also gives the major metabolite substituted in the K region, pyrene-4,5-dihydrodiol. The question arose whether extracellular enzymes like LiP, MnP, or laccase favor the further transformation of phenanthrene metabolites substituted in the C-1,2 and C-3,4 positions and are unable to attack metabolites substituted in the K region under ligninolytic conditions.

The results in this study are an extension of earlier studies on the fungal mineralization of phenanthrene and pyrene. Besides mineralization of PAHs, wood-decaying fungi produce metabolites with a higher water solubility and an enhanced chemical reactivity. Heitkamp et al. (15) have supposed that mono- or dihydroxylated PAH metabolites are substrates for bacteria and that degradation reactions in soil caused by indigenous microflora thus proceed more quickly. As recently reported (33), wood-decaying fungi like *K. mutabilis* and *A. aegerita* enhanced mineralization of pyrene in contaminated soil samples up to 75% in comparison with the indigenous microflora. Bacteria are not able to efficiently degrade PAHs with more than four aromatic rings. Fungi are able to metabolize (38) and to mineralize PAHs with more than four aromatic rings (36, 45). If the initial ring oxidation reaction and the formation of hydroxylated metabolites are the rate-limiting steps for PAH degradation, production of PAH metabolites is an important role of wood-decaying fungi for bioremediation of PAHs in polluted sites by indigenous soil microflora (45).

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REFERENCES

- Bezalel, L., Y. Hadar, P. P. Fu, J. P. Freeman, and C. E. Cerniglia. 1996. Metabolism of phenanthrene by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. 62:2547-2553.
- Bezalel, L., Y. Hadar, P. P. Fu, J. P. Freeman, and C. E. Cerniglia. 1996. Initial oxidation products in the metabolism of pyrene, anthracene, fluorene, and dibenzothiophene by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. 62:2554-2559.
- Blumer, M. 1976. Polycyclic aromatic compounds in nature. Sci. Am. 234: 34-45.

4. **Bogan, B. W., and R. T. Lamar.** 1995. One-electron oxidation in the degradation of creosote polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **61**:2631–2635.
5. **Bogan, B. W., and R. T. Lamar.** 1996. Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. *Appl. Environ. Microbiol.* **62**:1597–1603.
6. **Bumpus, J. A.** 1989. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **55**:154–158.
7. **Casillas, R. P., S. A. Crow, Jr., T. M. Heinze, J. Deck, and C. E. Cerniglia.** 1996. Initial oxidative and subsequent conjugative metabolites produced during the metabolism of phenanthrene by fungi. *J. Ind. Microbiol.* **16**:205–215.
8. **Cerniglia, C. E.** 1992. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* **3**:351–368.
9. **Cerniglia, C. E.** 1993. Biodegradation of polycyclic aromatic hydrocarbons. *Curr. Opin. Biotechnol.* **4**:331–338.
10. **Cerniglia, C. E., and M. A. Heitkamp.** 1989. Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment, p. 41–68. *In* U. Varanasi (ed.), *Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment*. CRC Press, Boca Raton, Fla.
11. **Cerniglia, C. E., D. W. Kelly, J. P. Freeman, and D. W. Miller.** 1986. Microbial metabolism of pyrene. *Chem.-Biol. Interact.* **57**:203–216.
12. **Cerniglia, C. E., J. B. Sutherland, and S. A. Crow.** 1992. Fungal metabolism of aromatic hydrocarbons, p. 193–217. *In* G. Winkelmann (ed.), *Microbial degradation of natural products*. VCH Press, Weinheim, Germany.
13. **Hammel, K. E., B. Kalyanaraman, and T. K. Kirk.** 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[*p*]dioxins by *Phanerochaete chrysosporium* ligninase. *J. Biol. Chem.* **261**:16948–16952.
14. **Hammel, K. E., W. Z. Gai, B. Green, and M. A. Moen.** 1992. Oxidative degradation of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **58**:1832–1838.
15. **Heitkamp, M. A., J. P. Freeman, D. W. Miller, and C. E. Cerniglia.** 1988. Pyrene degradation by a *Mycobacterium* sp.: identification of ring oxidation and ring fission products. *Appl. Environ. Microbiol.* **54**:2556–2565.
16. **Hirano, T., H. Tanaka, and A. Enoki.** 1995. Extracellular substance from the brown-rot basidiomycete *Tyromyces palustris* that reduces molecular oxygen to hydroxyl radicals and ferric iron to ferrous iron. *Mokuzai Gakkaishi* **41**:334–341.
17. **Hofrichter, M., and W. Fritsche.** 1997. Depolymerization of low-rank coal by extracellular fungal enzyme systems. II. The ligninolytic enzymes of the coal-humic-acid-depolymerizing fungus *Nematoloma frowardii* b19. *Appl. Microbiol. Biotechnol.* **47**:419–424.
18. **In der Wiesche, C., R. Martens, and F. Zadrazil.** 1996. Two-step degradation of pyrene by white rot fungi and soil microorganisms. *Appl. Microbiol. Biotechnol.* **46**:653–659.
19. **Kirk, T. K., E. Schulze, W. J. Connors, L. F. Lorenz, and J. G. Zeikus.** 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. *Arch. Microbiol.* **117**:277–285.
20. **Lambert, M., S. Kremer, O. Sterner, and H. Anke.** 1994. Metabolism of pyrene by the basidiomycete *Crinipellis stipitaria* and identification of pyrene-quinones and their hydroxylated precursors in strain JK375. *Appl. Environ. Microbiol.* **60**:3597–3601.
21. **Lang, E., F. Nerud, E. Novotna, F. Zadrazil, and R. Martens.** 1996. Production of ligninolytic exoenzymes and ¹⁴C-pyrene mineralization by *Pleurotus* sp. in lignocellulose substrate. *Folia Microbiol.* **41**:489–493.
22. **Lange, B., S. Kremer, O. Sterner, and H. Anke.** 1994. Pyrene metabolism in *Crinipellis stipitaria*: identification of *trans*-4,5-dihydro-4,5-dihydroxypyrene and 1-pyrenylsulfate in strain JK364. *Appl. Environ. Microbiol.* **60**:3602–3607.
23. **Lange, B., S. Kremer, O. Sterner, and H. Anke.** 1995. Induction of secondary metabolism by environmental pollutants: metabolism of pyrene and formation of 6,8-dihydroxy-3-methylisocoumarin by *Crinipellis stipitaria* JK 364. *Z. Naturforsch. Sect. C* **50**:806–812.
24. **Lange, B., S. Kremer, O. Sterner, and H. Anke.** 1996. Metabolism of pyrene by basidiomycetous fungi of the genera *Crinipellis*, *Marasmius* and *Marasmiellus*. *Can. J. Microbiol.* **42**:1179–1183.
25. **Martens, R., and F. Zadrazil.** 1992. Screening of white rot fungi for their ability to mineralize polycyclic aromatic hydrocarbons in soil, p. 505–510. *In* J. Klein (ed.), *Preprints of the International Symposium on Soil Decontamination Using Biological Processes*. DECHEMA, Frankfurt, Germany.
26. **Martens, R., H.-G. Wetzstein, F. Zadrazil, M. Capelari, P. Hoffmann, and N. Schmeer.** 1996. Degradation of the fluoroquinolone enrofloxacin by wood-rotting fungi. *Appl. Environ. Microbiol.* **62**:4206–4209.
27. **Moen, M. A., and K. E. Hammel.** 1994. Lipid peroxidation by the manganese peroxidase of *Phanerochaete chrysosporium* is the basis for phenanthrene oxidation by the intact fungus. *Appl. Environ. Microbiol.* **60**:1956–1961.
28. **Morgan, P., S. T. Lewis, and R. J. Watkinson.** 1991. Comparison of abilities of white-rot fungi to mineralize selected xenobiotic compounds. *Appl. Microbiol. Biotechnol.* **34**:693–696.
29. **Paszczynski, A., and R. L. Crawford.** 1995. Potential for bioremediation of xenobiotic compounds by the white-rot fungus *Phanerochaete chrysosporium*. *Biotechnol. Prog.* **11**:368–379.
30. **Pothuluri, J. V., and C. E. Cerniglia.** 1994. Microbial metabolism of polycyclic aromatic hydrocarbons, p. 92–124. *In* G. R. Chaudhry (ed.), *Biological degradation and bioremediation of toxic chemicals*. Dioscorides Press, Portland, Ore.
31. **Ritschkoff, A.-C., and L. Viikari.** 1991. The production of extracellular hydrogen peroxide by brown-rot fungi. *Mater. Org.* **26**:157–167.
32. **Sack, U., and T. Günther.** 1993. Metabolism of PAH by fungi and correlation with extracellular enzymatic activities. *J. Basic Microbiol.* **33**:269–277.
33. **Sack, U., and W. Fritsche.** 1997. Enhancement of pyrene mineralization in soil by wood-decaying fungi. *FEMS Microbiol. Ecol.* **22**:77–83.
34. **Sack, U., T. M. Heinze, J. Deck, C. E. Cerniglia, M. C. Cazau, and W. Fritsche.** 1997. Novel metabolites in phenanthrene and pyrene transformation by *Aspergillus niger*. *Appl. Environ. Microbiol.* **63**:2906–2909.
35. **Sack, U., M. Hofrichter, and W. Fritsche.** Degradation of phenanthrene and pyrene by *Nematoloma frowardii*. *J. Basic Microbiol.*, in press.
36. **Sack, U., M. Hofrichter, and W. Fritsche.** 1997. Degradation of PAHs by manganese peroxidase of *Nematoloma frowardii*. *FEMS Microbiol. Lett.* **152**:227–234.
- 36a. **Sack, U., and M. Hofrichter.** Unpublished data.
37. **Sutherland, J. B., A. L. Selby, J. P. Freeman, F. E. Evans, and C. E. Cerniglia.** 1991. Metabolism of phenanthrene by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **57**:3310–3316.
38. **Sutherland, J. B., F. Rafii, A. A. Khan, and C. E. Cerniglia.** 1995. Mechanism of polycyclic aromatic hydrocarbon degradation, p. 269–306. *In* L. Y. Young and C. E. Cerniglia (ed.), *Microbial transformation and degradation of toxic organic chemicals*. Wiley-Liss, New York, N.Y.
39. **Tien, M., and T. K. Kirk.** 1983. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization and catalytic properties of a unique H₂O₂-requiring oxygenase. *Proc. Natl. Acad. Sci. USA* **81**:2280–2284.
40. **Upadhyay, R. C.** 1995. Metabolism of phenolic substances by *Pleurotus flabellatus* (Berk and Br.) Sacc. and *Agrocybe aegeria* (Brig.) Sing. Ph.D. thesis. Friedrich Schiller University, Jena, Germany.
41. **Vazquez-Duhalt, R., D. W. S. Westlake, and P. M. Fedorak.** 1994. Lignin peroxidase oxidation of aromatic compounds in systems containing organic solvents. *Appl. Environ. Microbiol.* **60**:459–466.
42. **Wariishi, H., K. Valli, and M. H. Gold.** 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. *J. Biol. Chem.* **267**:23688–23695.
43. **Wilson, S. C., and K. C. Jones.** 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. *Environ. Pollut.* **81**:229–249.
44. **Wolfenden, B. S., and R. L. Willson.** 1982. Radical-cations as reference chromatogens in kinetic studies of one-electron transfer reactions. *J. Chem. Soc. Perkin. Trans. II*:805–812.
45. **Wolter, M., F. Zadrazil, R. Martens, and M. Bahadir.** Degradation of eight high condensed EPA-PAHs by *Pleurotus* sp. Florida in solid wheat straw substrate. *Appl. Microbiol. Biotechnol.*, in press.
46. **Wunder, T., S. Kremer, O. Sterner, and H. Anke.** 1994. Metabolism of the polycyclic aromatic hydrocarbon pyrene by *Aspergillus niger* SK 9317. *Appl. Microbiol. Biotechnol.* **42**:636–641.